Propose title of the thesis

Role of Fetuin-A in pancreatic β-cell dysfunction

Submitted by:
Alpana Mukhuty
Cell Signalling Laboratory
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Supervisor: Dr. Rakesh Kundu
Department of Zoology
Visva-Bharati, Santiniketan

INTRODUCTION:

Type 2 diabetes (T2D) is now posing a major threat to human health. World Health Organization has cautioned that it is taking the shape of a global epidemic. Prior to the manifestation of T2D there occurs impaired ability of insulin to maintain glucose homeostasis. This is because of defects in insulin sensitivity which leads to insulin resistance that results responsive tissues and cells suffer from impairment of glycemic regulation. To search the possible cause for the development and pathogenesis of insulin resistance, hyperlipidemia and dyslipidemia have been identified as the leading factors (Kolterman et al., 1979 ; Le et al., 1985). Continuation of this state ultimately manifested in the form of T2D. Several reports indicated that oversupply of lipid increases circulatory level of free fatty acid (FFA) and that contributes to the development of insulin resistance (Boden 1997; McGarry 2002). Patients suffering from insulin resistance often show signs of abnormal lipid metabolism where excess of plasma FFA level is coincided with reduced insulin-stimulated glucose uptake (Boden 1997; Boden and Shulman 2002). Lowering of circulatory elevated FFA level in diabetic patients improves insulin sensitivity (Santomauro et al., 1999). More than 95% are suffering from T2D while only 3-5% are T1D among world diabetic population (Zimmet 1999). It is also necessary to remember that in T2D loss of insulin sensitivity is the major event.

Many authors have shown that it is the fatty acid (FA) of lipid which is mostly associated with insulin resistance (Boden 1997). Some authors indicated that long chain saturated FAs are major culprit (Ruddock et al 2008). The list of FAs later has been shortened to 3-5 FAs and these are palmitate, myristate, stearate (saturated FAs) andoleate (unsaturated FA) (Vessby et al., 2002; Haag and Dippenaar 2005). There may be others, but reports on circulatory lipid profile in diabetic patients showed prevalence of these FAs and research area in the field of insulin resistance has predominantly used these FAs to blunt insulin sensitivity (Santomauro et al., 1999; Hennes et al.,1990; Wyne 2003). As already stated insulin

resistance is primarily due to insulin signaling defects. These defects, in fact, may occur at various places from upstream to downstream and that include 1) insulin receptor (IR), 2) phosphatidylinositol 3-kinase (PI3K), phophoinositide-dependent kinase1 (PDK1) and Akt mediated glucose transporter 4 (Glut4) activation, and 3) interplay of novel protein kinase Cs (nPKCs). Besides insulin signaling pathway another area of research is getting prominence which is adipocyte function. This is because any defect in the functioning of adipocyte would be reflected in lipid imbalance which is the root cause for insulin resistance. Insulin resistance, the centre of pathogenesis in T2D, is indeed a vast area with umpteen number of research publications in every year. In this review we tried to briefly cover the advancement made so far in the above three areas to understand the mechanism of lipid inducedinsulin resistance.

REVIEW OF LITERATURE:

Inflammation is not a disease in itself, but rather a manifestationof a disease. Initially, it has beneficial effects such as preventingthe spread of infection or promoting regeneration. However, ifprolonged or excessive, it may exacerbate disease by tissuedestruction. It is likely that, in the case of islet inflammation intype 2 diabetes, similar phenomena occur. Indeed, the endocrinepancreas may adapt to conditions of increased insulindemand (as encountered in obesity and pregnancy) byincreasing its functional mass. This may be triggered by limitedhyperglycemic events that provoke β cell production of low concentrations of IL-1β followed by Fas upregulation (Maedler et al.,2002a; Maedler et al., 2006). At these concentrations of IL-1β and in the presence of FLICE-like inhibitory protein (FLIP), Fasengagement leads to b cell proliferation and enhanced functionvia NF-kB and pancreatic and duodenal homeobox 1 (PDX1)(Schumann et al., 2007).

In obese and insulin-resistant individuals, intake of largeamounts of carbohydrates leads to transiently elevated glucoselevels defined as impaired glucose tolerance. With time, this prediabetesstate may progress to chronic hyperglycemia (i.e., diabetes).Additionally, elevated concentrations of other circulatingnutrients, such as free fatty acids (FFAs), are observed in obesityand type 2 diabetes (Boden, 1997). In parallel to increasednutrient concentrations, inflammation is observed in all tissuesinvolved in energy homeostasis, including fat, muscle, liver,and islets, as well as in the nutrient-transporting blood vessels(Berk et al., 1990; Hotamisligil et al., 1993; Maedler et al.,2002b; Navarro-Gonza´ lez and Mora-Ferna´ ndez, 2008; Ridkeret al., 1997; Wellen and Hotamisligil, 2005).

TLRs are pattern recognition receptors of the innate immune system that can be stimulated by numerousbacterial wall products such as lipopolysaccaride (LPS)-containinglong-chain fatty acid moieties. Therefore, it has been postulatedthat FFAs directly bind to TLRs and thereby induce aproinflammatory response (Lee et al., 2001, 2004). Recently, the liver-derivedglycoprotein fetuin-A was identified as the endogenous ligandlinking FFA and TLR4, causing inflammation and insulin resistance(Pal et al., 2012). FetA interferes with insulin receptor signalling at the tyrosine kinase level (Srinivas et al., 1993). Recent evidence showed that FetA blocks insulin-stimulated GLUT4 translocation. This study also clarified that FetA interferes with downstream phosphorylation events in insulin receptor signalling without affecting the binding of insulin to the α subunit of the receptor (Goustin et al., 2013).

FetA may also promote insulin resistance by propagating a pro-inflammatory state. Macrophage migration into adipose tissue and polarization from an anti-inflammatory M2 subtype to a pro-inflammatory M1 subtype are important events that promote the low-grade inflammatory state observed in obesity (Lumeng et al., 2007). Adipocyte derived FetA was recently found to signal both of these events (Chatterjee et al., 2013).

The first evidence for an inflammatory process in the pancreatic islet arose from the observation that hyperglycemia induces β cell apoptosis (Donath et al., 1999). Unraveling the underlying mechanism (Figure 1), it turns out that high-glucose concentrations induce the Fas receptor, which is upregulated via glucose-induced IL-1β production (Maedler et al., 2001, 2002a, 2002b). Similarly, recent evidence shows that fatty acids also promote an inflammatory response (Boni-Schnetzler et al., 2009; Ehses et al., 2007). In support of insulitis in type 2 diabetes, elevated numbers of immune cells have been detected in islets of patients with type 2 diabetes and also in animal models ,with increased levels of cytokines and chemokines (Boni-Schnetzler et al., 2008; Ehses et al., 2007; Richardsonet al., 2009). Every animal model of type 2 diabetes investigated till date displays islet immune cell infiltration (Ehses et al., 2007, 2009a). Furthermore, a strong argument for the occurrence of an inflammatory process in islets is the well-described fibrosis observed in tissue sections of patientswith type 2 diabetes, characterized by amyloid deposits. Indeed, fibrosis is a hallmark of the end stage of a chronic inflammatory process. Of interest is the predominant role of IL-1β, which is upregulated in islets of patients with type 2 diabetes (Boni-Schnetzler et al., 2008; Maedler et al., 2002b). This master cytokine regulates numerous other cytokines and chemokines (Dinarello, 2009; Ehses et al., 2009b). Thereby, it contributes to the recruitment of immune cells implementing a broad inflammatory response.

It has been reported that FetA or TLR4 knockout mice are protected from high-fat diet(HFD)-induced insulin resistance (Pal et al., 2012). But it remains unclear whether FetA can mediate FFA-TLR crosstalk resulting in lipotoxicity-mediated inflammatory injury in β cells.

KNOWLEDGE GAP IN β CELL DESTRUCTION:

(i) Role of Fetuin-A in progressive detoriation of β cell function
(ii) Fetuin-A mediated lipotoxicity in β cells through TLR4 signalling pathway
(iii) Fetuin-A induced macrophage migration to β cells and polarization of M2 macrophages to proinflamatory M1 subtype
(iv) Role of AMPK activation in intervention induced lowering of circulating Fetuin-A

OBJECTIVE:

Based on the above observation the following objectives have been proposed in the present work:

1. To study the role of Fetuin-A in progressive dysfunction in pancreatic β cell lines (MIN6 and BTC6)
2. To study the Fetuin-A mediated β cell dysfunction in HFD mice model

3. To study theFetuin-A mediated lipotoxicitythrough TLR4 signalling pathway by the complex formation of FFA-Fetuin-A-TLR4 in MIN6 and βTC6 cells
4. To elucidate the Fetuin-A induced macrophage migration to β cells and polarization of M2 macrophages to proinflammatory M1 macrophages in MIN6 and βTC6 cells

MATERIALS AND METHODS:

Cell culture

The mouse islet cell lines βTC6 and MIN6 will be obtained from the NCCS,Pune. Cells will be culturedwith incomplete medium: high-glucose Dulbecco's modified Eagle'smedium (HDMEM, 25 mmol/L glucose),2 mmol/L L-glutamine at 37 °C and 5% CO2.All reagents will be purchased from Gibco (Carlsbad, CA, USA). Cellsat passages 26–35 will be used for all experiments.

Animal model

Five-to-six weeksold, male Swiss albino mice (70 ± 8 g) will be obtained. After a 1-week acclimation period, the animals will be weighed and measured, and randomly dividedinto two groups: those receiving normal control diet (n = 20), andthose receiving a high-fat diet (HFD, n = 20).HFD mice will receive a HFD (Research Diets;D12492; 60% calories from fat, 20% calories from protein, 20% calories from carbohydrate) for 8 weeks, to induce obesity. Diet-induced obesity isdefined as an average increase, above control, in weight and Lee'sindex (Wei et al., 2011) [Lee's index = body weight (g)1/3 × 103/body length (cm)] of 15% and 1.5%, respectively. Animals will be housed, five mice per cage, in a temperature- and humiditycontrolledroom (20 ± 26 ℃, 40–60% humidity), with a set 12-hourlight–dark cycle. The body weight and length will be recorded once per week for the duration of the study.

Serum glucose , lipid and insulin measurements:

Mice blood samples will be prepared by venous sampling. Serum glucose and lipid will be measured by Onetouch Ultra blood glucose monitoring system (LifeScan,Milpitas, USA) and automatic biochemistry analyzer, respectively. Serum insulin was measured by insulin ELISA kit (Linco, St Charles, USA).

Dose and time kinetics in the dysfunction of β cells

The β cells will be cultured and subjected to different doses of Fetuin-A (1µg/ml, 2µg/ml, 4µg/ml,10 µg/ml,20µg/ml) and at different times(2h, 6h,10h, 14h, 20h, 24h, 30h, 36h, 40h, 48h) to study the dose-dependant and time-dependant β-cell dysfunction in-vivo and in-vitro.

β cell isolation

Briefly, at the end of treatment (8 weeks), mice from the control and HFD group will anesthetized by injection. Collagenase solution (0.5 mg/ml) will be injected through the common bile duct. The expanded pancreatic islet will be then then isolated,put into culture flask with 6 ml Krebs solution, and digested at 38 C for 10 min. The liquid will be sifted by passing through a stainless steel net (600 mm), and then ice-cold Krebs solution will be added to stopthe digestion. The mixture will be centrifuged at 250 g for 1 min at 4 C, and the supernatant will be discarded. This process will be repeated twice. The precipitate will be

resuspended in 25%Ficoll solution in a centrifuge tube, and then 23%, 20%, and 11% Ficoll solutions will be added carefully on top of each previous layer. After being centrifuged at 500 g for 15 min at4C, the interface between 23% and 20% Ficoll solutions orbetween 20% and 11% Ficoll solutions will be obtained andwashed three times with Krebs solution. The obtained pancreaticislet will be identified by dithizone.

Protein extraction and western blotting

Whole cell extracts will be prepared for respectiveWestern blot analysis .Islets isolated from each mice pancreaswill be washed twice withPBS and lysed in radioimmune precipitation assay buffer containing50mM Tris (pH 7.4), 150mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS with protease inhibitors on ice for 30 min. Protein concentrations will be estimated by the Lowry's method . Ninety micrograms of protein samples will be separatedvia SDS–polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes (Sigma). Membranes will be blockedwith 5% fat-free milk in Tris-buffered saline solution (TBS) containing0.1% Tween-20 and incubated overnight at 4 °C with anti-TLR4(SC-16240, Santa Cruz), anti-GRP40 (sc-32905, Santa Cruz), anti-PDX-1 (sc-47267, Santa Cruz), anti-p-JNK (4671, CST), anti-p-NF-κB p65 (sc-135768, Santa Cruz), anti-cleaved caspase-3 (sc-7272,Santa Cruz) or anti-β-actin antibody (sc-47778, Santa Cruz). Then the membranes will be incubated with Horseradish peroxidase–labeled secondary antibody (1:1000) for 3 h at room temperature. Protein signals will be visualized using the enhanced chemiluminescencedetectionsystem.

Apoptosis analysis by TUNEL analysis and flow cytometry

The TUNEL will be done by Dead-EndTM Fluorometric TUNEL System(Promega, Madison, Wisconsin). PI stains both apoptotic andnonapoptotic cells red. Fluorescein-12-dUTP incorporation resultsin localized green fluorescence within the nucleus of apoptotic cellsonly. Islet cells will be seeded in 6-well plates and treated as indicated. The cells will be collected after digestion with 0.25% EDTA-free trypsin and centrifuged at 10,000 rpm for 5 min. The cell pellet will then be then washed twice with cold PBS, and the AnnexinV-FITC/PI Apoptosis Detection Kit (BD Biosciences) will beused to assess cell death. The cells will be harvested by centrifugation, and labeling will be carried out by serial addition of Annexin V and PI in the dark, according to the manufacturer's instructions. Cell apoptosis will beanalyzed on a Cytomics™ FC500 Flow Cytometer(Beckman Coulter, Brea, CA, USA). Annexin V-FITC(+)/PI(−) cells will be scored as apoptotic, and Annexin V-FITC(+)/PI(+) (double stained) cells will be considered either necrotic or late apoptotic.

Protein inhibitors intervention

To observe the effect of TLR4, JNK, and NF-κB activity on thelipotoxicity effect of PA + FetA in β-cell, β cells will be culturedin 6-well plates with incomplete medium (HDMEM + 2 mmol/L L-glutamine) until the cells reach 60% confluence, andthen they will be pre-treated with 3 μmol/L TLR4 inhibitor (CLI-095, dissolvedin 0.1% DMSO (v/v); Sigma–Aldrich), 25 μmol/L JNK inhibitor(SP600125, dissolved in 0.1% DMSO (v/v); Sigma–Aldrich) or 20 μmol/L NF-κB inhibitor (PDTC, dissolved in 0.1% DMSO (v/v);Sigma–Aldrich) with

HDMEM + 2 mmol/L L-glutamine respectivelyfor 24 h. After reaching 60% confluency the medium will be removed and the cells will be washedtwice with PBS. Then the cells will be exposed in 0.5 mmol/L PA + 20 μg/MlFetA + 3 μmol/L CLI-095 (or 25 μmol/L SP600125 or 20 μmol/L PDTC)in HDMEM without FBS or BSA for 24 h. Cells exposed to medium alone will be used as control.

Glucose stimulated insulin secretion (GSIS)

β cells will be seeded in 6-well plates and treated as indicatedabove.Then cells will be washed twice with glucose-freeKrebs–Ringer bicarbonate buffer (KRBB) (pH 7.4) and pre-incubatedat 37 °C for 30 min with the glucose-free KRBB. Cells will bethenwashed once with glucose-free KRBB and incubated for 60 min at37 °C in KRBB containing 0 or 20 mmol/L glucose. Aliquots of supernatantwere collected and stored at 20 °C for insulin determination by ELISA kit (Cusabio, intra- and interassay coefficientsof variation of <8% and <10%, respectively).

Immunohistolochemistry

The pancreas will be isolated from both the control and HFD groups. The pancreas will be fixed in Bouin's fluid followed by dehydration in ascending grades of alcohol. Paraffin embedded tissues will be cut at appropriate thickness using a Microtome and affixed on glass slides for further observation. The sections will be deparaffinised in xylene and rehydrated with descending grades of alcohol.For immunohistochemicallabeling, tissue sections will be sequentially treated with blocking solution (1–2 h), incubated in primary antibodies overnight (4°C), and incubated with appropriate Alexafluor-conjugated secondary antibodies (1–2 h) the following day . Draq5 (Biostatus Limited) at 1:3000 dilution will be used as a nuclear counterstain. Immunofluorescentlylabeled M1 and M2 cells will be quantified under high-power magnification with wide field-fluorescence. Macrophage specificity will be confirmed by double labelling with macrophage markers (tomato lectin) and M1 or M2 markers.

Transwell system of co-culture

Indirect co-cultures will be performed using adipocytes in 0.4 μm pore size cell culture inserts and then place them in 6-well plates containing β cells. Co-cultures will be incubated for 6 hr with or without FA and then the

Cell migration assay

The RAW264.7 cell migration assay was performed in a Boyden chamber system (Millipore QCM 24-well colorimetric cell migration assay kit) by the addition of FetAtreated β cells in the lower chamber.

ELISA

Cytokine levels will be measured in the cell culture medium from both control and FA treated β cells using TNFα and IL-6 ELISA kits and FetA ELISA kits following manufacturer's protocol.

References

Alessi, D.R. and Cohen, P. (1998) Mechanism of activation and function of protein kinase B. *Current Opinion in Genetics and Development* **8,** 55-62

Bastard, J.P., Jardel, C., Bruckert, E., Blondy, P., Capeau, J. and Laville, M. (2000) Elevated levels of interleukin 6 are reduced in serum and subcutaneous adipose tissue of obese women after weight loss. *Journal of Clinical Endocrinology and Metabolism* **85,** 3338–3342.

Beeson, M., Sajan, M.P., Dizon, M., Grebenev, D., Gomez-Daspet, J., Miura, A.,Kanoh, Y., Powe, J., Bandyopadhyay, G., Standaert, M.L. and Farese, R.V.(2003) Activation of protein kinase C-zeta by insulin and phosphatidylinositol-3,4,5-(PO4)3 is defective in muscle in type 2 diabetes and impaired glucose tolerance: amelioration by rosiglitazone and exercise. *Diabetes* **52,** 1926-1934

Bendtzen, K., Mandrup-Poulsen, T., Nerup, J., Nielsen, J.H., Dinarello , C.A. and Svenson,M.(1986) Cytotoxicity of human pI 7 interleukin-1 for pancreatic islets of Langerhans. *Science* **232,**1545–1547

Berk, B.C., Weintraub, W.S., and Alexander, R.W. (1990) Elevation of C-reactive protein in ''active'' coronary artery disease. *American Journal of Cardiology* **65,** 168–172

Boden, G., Chen, X., Ruiz, J., White, J.V. and Rossetti, L. (1994) Mechanisms of fatty acid-induced inhibition of glucose uptake. *Journal of Clinical Investigation* **93,** 2438-2446

Boden, G. (1997) Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. *Diabetes* **46,** 3–10

Boden. G. and Shulman, G.I. (2002) Free fatty acids in obesity and type 2 diabetes: defining their role in the development of insulin resistance and beta-cell dysfunction.*European Journal of Clinical Investigation* **32,** 14-23

Boni-Schnetzler, M., Ehses, J.A., Faulenbach, M. and Donath, M.Y. **(**2008) Insulitis in type 2 diabetes. *Diabetes Obesity and Metabolism* **10,** 201–204

Boni-Schnetzler, M., Thorne, J., Parnaud, G., Marselli, L., Ehses, J.A., Kerr- Conte, J., Pattou, F., Halban, P.A., Weir, G.C., and Donath, M.Y. (2008) Increased interleukin (IL)-1beta messenger ribonucleic acid expression in beta -cells of individuals with type 2 diabetes and regulation of IL-1beta in human islets by glucose and autostimulation. *Journal of Clinical Endocrinology and Meta*bolism **93,** 4065–4074

Boni-Schnetzler, M., Boller, S., Debray, S., Bouzakri, K., Meier, D.T., Prazak, R., Kerr-Conte, J., Pattou, F., Ehses, J.A., Schuit, F.C., and Donath, M.Y. (2009) Free fatty acids induce a proinflammatory response in islets via the abundantly expressed interleukin-1 receptor I. *Endocrinology* **150,** 5218– 5229

Brooks-Worrell, B., Tree, T., Mannering, S.I., Durinovic-Bello, I., James, E.,Gottlieb, P., Wong, S., Zhou, Z., Yang, L., and Cilio, C.M. T-Cell Workshop Committee, Immunology of Diabetes Society. (2011) Comparison of cryopreservation methods on T-cell responses to islet and control antigens from type 1 diabetic patients and controls. *Diabetes Metabolism Research and Rev*iews **27,** 737–745

Cacicedo, J.M., Yagihashi, N., Keaney, J.F., Ruderman, N.B. and Ido, Y. (2004) AMPK inhibits fatty acid-induced increases in NF-kappaB transactivation in cultured human umbilical vein endothelial cells. *Biochemical and Biophysical Research Communications* **324,**1204–1209

Chatterjee, P., Seal, S., Mukherjee, S., Kundu, R., Mukherjee, S., Ray, S., Mukhopadhyay, S., Majumdar, S.S., and Bhattacharya, S.(2013) Adipocyte fetuin-a contributes to macrophage migration into adipose tissue and polarization of macrophages. *Journal of Biological Chemistry* **288,** 28324-28330

Dinarello, C.A. (2009) Immunological and inflammatory functions of the interleu family. *Annual Review of Immunology* **27,**519–550

Donath, M.Y., Gross, D.J., Cerasi, E., and Kaiser, N. (1999) Hyperglycemia induced beta-cell apoptosis in pancreatic islets of Psammomys obesus during development of diabetes. *Diabetes* **48,** 738–744

Ehses, J.A., Perren, A., Eppler, E., Ribaux, P., Pospisilik, J.A., Maor-Cahn, R., Gueripel, X., Ellingsgaard, H., Schneider, M.K., and Biollaz, G. (2007) Increased number of islet-associated macrophages in type 2 diabetes. *Diabetes* **56,** 2356–2370

Ehses, J.A., Ellingsgaard, H., Bo¨ni-Schnetzler, M., and Donath, M.Y. (2009a) Pancreatic islet inflammation in type 2 diabetes: from alpha and beta cell compensation to dysfunction. *Archives of Physiology and Biochemtry* **115,** 240–247

Goldfine, A.B., Fonseca, V.and Shoelson, S.E.(2011) Therapeutic approaches to target inflammation in type 2 diabetes. *Clinical Chemistry* **57,** 162–167

Haber, E.P., Procopio, J., Carvalho, C.R., Carpinelli, A.R., Newsholme, P. And Curi, R. (2006) New insights into fatty acid modulation of pancreatic β-cell function. *International Review of Cytology* **248,** 1–41

Holzer, R.G., Park, E.J., Li, N., Tran, H., Chen, M., Choi, C., Solinas, G., and Karin, M. (2011). Saturated fatty acids induce c-Src clustering within membrane subdomains, leading to JNK activation. *Cell* **147,** 173–184

Homo-Delarche, F., Calderari, S., Irminger, J.C., Gangnerau,M.N.,Coulaud,J., Rickenbach, K., Dolz, M., Halban, P., Portha, B., Serradas, P.(2006) Islet inflammation and fibrosis in a spontaneous model of type 2 diabetes,the GK rat. *Diabetes* **55,**1625–1633

Hotamisligil, G.S., Shargill, N.S., and Spiegelman, B.M. (1993) Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* **259,** 87–91

Itani, S.I., Ruderman, N.B., Schmieder, F. and Boden, G. (2002) Lipid-induced insulin resistance in human muscle is associated with changes in diacylglycerol, protein kinase C, and IkappaB-alpha. *Diabetes* **51,** 2005-2011

Jung, T.W., Youn, B.S., Choi, H.Y., Lee, S.Y., Hong, H.C. and Yang, S.J. (2013) Salsalate and adiponectin ameliorate hepatic steatosis by inhibition of the hepatokine fetuin-A. *Biochemical Pharmacology* **86,** 960-969

Kahn, B.B. and Flier, J.S.(2000) Obesity and insulin resistance. *Journal of Clinical Investigation* **106,** 473–481

Kharroubi, I., Ladriere, L., Cardozo, A.K., Dogusan, Z., Cnop, M. and Eizirik, D.L. (2004) Free fatty acids and cytokines induce pancreatic β-cell apoptosis by different mechanisms: role of nuclear factor-κB and endoplasmic reticulum stress. *Endocrinology* **145,** 5087–5096

Koshkin, V., Dai, F.F., Robson-Doucette, C.A., Chan, C.B. and Wheeler, M.B.(2008) Limited mitochondrial permeabilization is an early manifestation of palmitate-induced lipotoxicity in pancreatic β-cells. *Journal of Biological Chemistry* **283,** 7936–7948

Kupriyanova, T.A., Kandror, K.V.(1999) Akt-2 binds to Glut4-containing vesicles and phosphorylates their component proteins in response to insulin. *Journal of Biological Chemistry* **274,** 1458-1464

Laemmli, U.K. (1970) Cleavage of structural structural proteins during the assembly of the head of bacteriophage T4. *Nature* **27,** 680-685

Lee, J.Y., Sohn, K.H., Rhee, S.H., and Hwang, D. (2001) Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4. *Journal of Biological Chemistry* **276,** 16683–16689

Lee, J.Y., Zhao, L., Youn, H.S., Weatherill, A.R., Tapping, R., Feng, L., Lee, W.H., Fitzgerald, K.A., and Hwang, D.H. (2004) Saturated fatty acid activates but polyunsaturated fatty acid inhibits Toll-like receptor 2 dimerized with Tolllike receptor 6 or 1. *Journal of Biological Chemistry* **279,** 16971–16979

Lorenzo, A., Razzaboni, B., Weir, G.C. and Yankner, B.A.(1994) Pancreatic islet cell toxicity of amylin associated with type-2 diabetes mellitus. *Nature* **368,**756–760

Lumeng , C.N., Bodzin, J.L., and Saltiel, A.R.(2007) Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *Journal of clinical investigation* **117,** 175-184

Maedler, K., Spinas, G.A., Lehmann, R., Sergeev, P., Weber, M., Fontana, A., Kaiser, N., and Donath, M.Y. (2001) Glucose induces beta-cell apoptosis via upregulation of the Fas receptor in human islets. *Diabetes* **50,** 1683–1690

Maedler, K., Fontana, A., Ris, F., Sergeev, P., Toso, C., Oberholzer, J., Lehmann, R., Bachmann, F., Tasinato, A., and Spinas, G.A. (2002a) FLIP switches Fas-mediated glucose signaling in human pancreatic b cells from apoptosis to cell replication. *Proceedings of the National Academy of Sciences, USA* **99,** 8236–8241

Maedler, K., Sergeev, P., Ris, F., Oberholzer, J., Joller-Jemelka, H.I., Spinas,G.A., Kaiser, N., Halban, P.A., and Donath, M.Y. (2002b) Glucose-induced beta cell production of IL-1beta contributes to glucotoxicity in human pancreatic islets. *Journal of Clinical Investigation* **110,** 851–860

Maedler, K., Oberholzer, J., Bucher, P., Spinas, G.A. and Donath, M.Y. (2003) Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic β-cell turnover and function. Diabetes 52:726–733

Mandrup-Poulsen, T. (1996) The role of interleukin-1 in the pathogenesis of IDDM. *Diabetologia* **39,**1005–1029

Marselli, L., Sgroi, D.C.,Thorne, J., Dahiya, S., Torri, S.,Omer,A., Del Prato, S., Towia, L., Otu, H,H., Sharma, A., Bonner-Weir, S., Marchetti, P., Weir, G.C.(2007) Evidence of inflammatory markers in β-cells of type 2 diabetic subjects. 43rd Annual Meeting of the European Association for the Study of Diabetes, Amsterdam, The Netherlands, 2007, 0418 (Abstract)

Martin, S., Millar, C.A., Lyttle, C.T., Meerloo, T., Marsh, B.J., Gould, G.W. and James ,D.E.(2000) Effects of insulin on intracellular GLUT4 vesicles in adipocytes: evidence for a secretory mode of regulation. *Journal of Cell Science* **113,** 3427-3438

Navarro-Gonza´lez, J.F., and Mora-Ferna´ndez, C. (2008) The role of inflammatory cytokines in diabetic nephropathy. *Journal of the American Society of Nephrology*. **19,** 433–442

Nguyen, M.T., Favelyukis, S., Nguyen, A.K., Reichart, D., Scott, P.A., Jenn, A., Liu-Bryan, R., Glass, C.K., Neels, J.G. and Olefsky,J.M. (2007) A subpopulation of macrophages infiltrates hypertrophic adipose tissue and is activated by free fatty acids via Toll-like receptors 2 and 4 and JNK-dependent pathways. *Journal of Biological Chemistry* **282,** 35279–35292

Pal, D., Dasgupta, S., Kundu, R., Maitra, S., Das, G., Mukhopadhyay, S., Ray, S., Majumdar, S.S., and Bhattacharya, S. (2012) Fetuin-A acts as an endogenous ligand of TLR4 to promote lipid-induced insulin resistance. *Nature Medicine* **18,** 1279-1285

Park, J.S., Svetkauskaite, D., He, Q., Kim, J.Y., Strassheim, D., Ishizaka, A.,and Abraham, E. (2004). Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. *Journal of Biological Chemistry* **279,** 7370–7377

Pan, D.A., Lillioja, S., Kriketos, A.D., Milner, M.R., Baur, L.A., Bogardus, C., Jenkins, A.B. and Storlien, L.H. (1997) Skeletal muscle triglyceride levels are inversely related to insulin action. *Diabetes* **46,** 983-988

Peraldi, P. and Spiegelman, B.(1998) TNFa and insulin resistance: summary and future prospects. *Molecular Cell Biochemistry* **182,** 169–175

Pessin, J.E., Thurmond, D.C., Elmendorf, J.S., Coker, K.J. and Okada, S. (1999) Molecular basis of insulin-stimulated GLUT4 vesicle trafficking. Location! Location!Location! *Journal of Biological Chemistry* **274,** 2593-2596

Pessin, J.E. and Saltiel, A.R. (2000) Signaling pathways in insulin action: molecular targets of insulin resistance. *Journal of Clinical Investigation* **106,** 165-169

Reynoso, R., Salgado, L.M. and Calderón, V. (2003) High levels of palmitic acid lead to insulin resistance due to changes in the level of phosphorylation of the insulin receptor and insulin receptor substrate-1. *Molecular Cell Biochemistry* **246,** 155-162

Richardson, S.J., Willcox, A., Bone, A.J., Foulis, A.K., and Morgan, N.G. (2009) Islet-associated macrophages in type 2 diabetes. *Diabetologia* **52,** 1686–1688

Ridker, P.M., Cushman, M., Stampfer, M.J., Tracy, R.P., and Hennekens, C.H.(1997)Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. *The New England Journal of Medicine:Research and Reviews* **336,** 973–979

Rosen, O.M. (1987) After insulin binds. *Science* **237,** 1452-1458

Saltiel, A.R. and Kahn, C.R.(2001) Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* **414,** 799-806

Senn, J.J. (2006) Toll-like receptor-2 is essential for the development of palmitate-induced insulin resistance in myotubes. *Journal of Biological Chemistry* **281,** 26865–26875

Shi, H., Kokoeva,M.V.,Inouye, K., Tzameli, I., Yin. H. and Flier, J.S. (2006) TLR4 links innate immunity and fatty acid-induced insulin resistance.*Journal of Clinical Investigation* **116,** 3015–3025

Shulman, G.I.(2000) Cellular mechanisms of insulin resistance. *Journal of Clinical Investigation* **106,** 171-176

Song, M.J., Kim, K.H., Yoon, J.M. and Kim, J.B. (2006) Activation of Toll-like receptor 4 is associated with insulin resistance in adipocytes. *Biochemistry Biophysics Research Communities* **346,** 739–745

Song, Z., Deaciuc, I., Zhou, Z., Song, M., Chen, T. and Hill, D. (2007) Involvement of AMP activated protein kinase in beneficial effects of betaine on high-sucrose diet induced hepatic steatosis. *American Journal of Physiology Gastrointestinal and Liver Physiology* **293,** 894–902.

Srinivas, P., Wagner, A.S., Reddy, L.V., Deutsch, D., Leon, M.A., and Goustin, A.S. (1993) Serum alpha 2-HSglycoprotein is an inhibitor of the human insulin receptor at the tyrosine kinase level. *Molecular Endocrinology* **7,** 1445-1455

Staiger, H., Staiger, K., Stefan, N., Wahl, H.G., Machicao, F., Kellerer, M. and Haring, H.U. (2004) Palmitate-induced interleukin-6 expression in human coronary artery endothelial cells. *Diabetes* **53,** 3209–3216

Unger, R.H. (1995) Lipotoxicity in the pathogenesis of obesity-dependent NIDDM. Genetic and clinical implications. *Diabetes* **44,**863–870

Weir, G.C., Leahy, J.L., Bonner-Weir. S. (1986) Experimental reduction of B-cell mass: implications for the pathogenesis of diabetes. *Diabetes Metabolism and Reviews* **2,**125–161

Wellen, K.E. and Hotamisligil, G.S. (2003) Obesity-induced inflammatory changes in adipose tissue. *Journal of Clinical Investigation* **112,** 1785–1788.

Wellen, K.E., and Hotamisligil, G.S. (2005) Inflammation, stress, and diabetes. *Journal of Clinical Investigation* **115,** 1111–1119

Wen, H., Gris, D., Lei, Y., Jha, S., Zhang, L., Huang, M.T., Brickey, W.J., and Ting, J.P. (2011). Fatty acid-induced NLRP3-ASC inflammasome activation interferes with insulin signaling. *Nature Immunology* **12,** 408–415

Wentworth, J.M., Fourlanos, S., and Harrison, L.C. (2009) Reappraising the stereotypes of diabetes in the modern diabetogenic environment. *Nature Reviews Endocrinology* **5,** 483–489

Wong, S.W., Kwon, M.J., Choi, A.M., Kim, H.P., Nakahira, K., and Hwang, D.H. (2009). Fatty acids modulate Toll-like receptor 4 activation through regulation of receptor dimerization and recruitment into lipid rafts in a reactive oxygen species-dependent manner. *Journal of Biological Chemistry* **284,** 27384–27392

Yang, J., Maika, S., Craddock, L., King, J.A. and Liu, Z.M. (2008) Chronic activation of AMPactivated protein kinase-alpha1 in liver leads to decreased adiposity in mice. *Biochemical and Biophysical Research Communications* **370,** 248–53

Yin, J., Peng, Y., Wu, J., Wang, Y., and Yao, L. (2014) Toll-like receptor 2/4 links to free fatty acid-induced inflammation and β-cell dysfunction. *Journal of Leukocyte Biology* **95,** 47-52

Yu, C., Chen, Y., Cline, G.W., Zhang, D., Zong, H., Wang, Y., Bergeron, R., Kim, J.K., Cushman,W., Cooney, G.J., Atcheson, B., White, M.F., Kraegen, E.W. and Shulman, G.I.(2002) Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. *Journal of Biological Chemistry* **277,** 50230-50236